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Temporal and Histologic Relationships of Proliferating Cell Nuclear Antigen and Human Papillomavirus Type 11 in the Mouse Xenograft System

Darron R. Brown, Linda Pratt, Kenneth H. Fife, and Janine T. Bryan

Department of Medicine, Division of Infectious Diseases (D.R.B., L.P., K.H.F.), Departments of Pathology (K.H.F.) and Microbiology and Immunology (K.H.F., J.T.B.), Indiana University School of Medicine, and Richard L. Roudebush Veterans Administration Medical Center (D.R.B.), Indianapolis, Indiana

Proliferating cell nuclear antigen (PCNA) is an accessory protein of DNA polymerase delta. This protein is associated with cell cycle progression and can be detected in the replicating cells of normal tissues. Condylomata acuminata are benign epithelial tumors caused by infection with human papillomaviruses and are characterized by abnormal cell proliferation. The athymic mouse xenograft model of HPV 11 infection was used to test the hypothesis that PCNA is induced early in the course of HPV 11 infection and to study the temporal and histologic relationships between detection of PCNA and HPV DNA. Human foreskin tissue was infected with HPV 11 and implanted under the renal capsules of 10 athymic mice. Pairs of mice were sacrificed every week beginning four weeks after implantation. HPV DNA was detected in sections of foreskin implants by in situ hybridization. PCNA was as or more abundant in implants removed at earlier time points than at later time points, whereas HPV DNA became increasingly more abundant with time. PCNA was detected only in basal cells in areas of histologically normal epithelium that were also negative for HPV DNA. In contrast, PCNA was present throughout the epithelium in regions that were HPV DNA-positive. HPV DNA was detected only in differentiated epithelial cells in implants removed at all five time points, but in HPV DNA-positive regions, PCNA was detected with equal intensity in differentiated and undifferentiated cells. The foci of PCNA-positive cells were well demarcated and were larger than, but included, the foci of HPV DNA-positive cells. PCNA may be induced maximally in differentiated epithelium by HPV 11 prior to significant HPV DNA replication.

KEY WORDS: HPV 11 infection, proliferating cell nuclear antigen (PCNA)

INTRODUCTION

Human papillomaviruses infect the genital tract, causing epithelial lesions that may be benign or malignant. Human papillomavirus types 6 and 11 (HPV 6 and HPV 11) cause the majority of condylomata acuminata lesions and are considered "low-risk" types for development of genital malignancy [zur Hausen and de Villiers, 1994].

Normal stratified epithelium is composed of four histologically distinct layers. Stem cells with proliferation potential are present in the basal layer. A progression of differentiation occurs through the spinous, granular, and corneal layers. Condylomata acuminata are benign epithelial tumors caused by infection with HPV and are characterized by branching papillae with fibrovascular cores and marked epithelial thickening primarily in the spinous layer [Crum, 1994]. The HPV genome is maintained in the basal cells of condylomata acuminata lesions as low-copy-number extrachromosomal plasmids, with vegetative viral DNA amplification occurring only in the differentiated cells of the upper epithelial layers [Stoler et al., 1990]. HPV types 16 and 18 characterize the "high-risk" types and are present in most high-grade intraepithelial lesions of the cervix and other genital sites [Howley, 1991; McCance et al., 1985]. These lesions also contain abnormally thickened squamous epithelium. Most invasive carcinomas contain integrated HPV DNA, generally at lower viral copy numbers than the low-risk HPV types present in condylomata acuminata [Stoler et al., 1992].

Proliferating cell nuclear antigen (PCNA) is a 36 kDA accessory protein of DNA polymerase delta [Almendral et al., 1987; Bravo et al., 1987]. PCNA is not detected in quiescent epithelial cells but is induced during cell cycle transition from G_0 to G_1 and further in-

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Address reprint requests to Darron R. Brown, Indiana University School of Medicine, 545 Barnhill Drive, Indianapolis, IN 46202.

creased during S phase [Morris and Mathews, 1989]. PCNA is believed to form a homotrimer structure encircling DNA, providing a platform for attachment of DNA polymerase delta [Krishna et al., 1994]. PCNA can be detected in the proliferative cells of normal tissues. PCNA is normally detected only in basal cells of squamous epithelium [Hall et al., 1990]. Genital HPV infection has previously been shown to be associated with PCNA induction. Demeter et al. [1994] showed that PCNA could be identified in squamous cell carcinomas of cervix and vulva and in condylomata acuminata (in areas of high viral copy number).

This study was designed to test the hypothesis that PCNA is induced early in the course of HPV 11 infection. The mouse xenograft model of HPV 11 infection was used to study the temporal and histological relationship between HPV 11 DNA and PCNA [Kreider et al., 1987]. Previous studies of PCNA detection in HPV infection have examined condylomata acuminata that had been present for unknown periods of time, making any studies of temporal relationships impossible. Because it is thought that the basal layer of epithelial cells are infected initially with HPV, we expected to detect PCNA mainly in these same cells and not in differentiated cells early in the course of infection. HPV 11-infected human foreskin implants that had been removed from athymic mice at five time points were used to prepare paraffin-embedded tissue sections. These sections were analyzed for histologic abnormalities consistent with HPV infection, HPV 11 DNA, and PCNA.

MATERIALS AND METHODS

HPV 11-Infected Human Foreskin Implants Grown in Athymic Mice

Human foreskin tissue inoculated with purified HPV 11 virions was implanted under the renal capsules of athymic mice (nu/nu on a Balb/c background), as previously described [Brown et al., 1994; Kreider et al., 1987]. To summarize, a freshly acquired human foreskin was placed on a sterile field, and dermal tissue was removed from the epidermis. A single foreskin was used for all 10 mice. The epidermal tissue was cut into fragments of ~4 mm^2 and placed in medium containing penicillin and streptomycin. Purified HPV 11 virions in PBS were added and incubated for 90 minutes at 37°C. Infected foreskin fragments were implanted under the renal capsule of each kidney (i.e., two implants per mouse).

Pairs of mice were killed 4, 5, 6, 7, and 8 weeks after infection and implantation. Foreskin implants were removed from mice, weighted, and split into two equal fragments. The first fragment was used to extract DNA for other experiments. The second fragment was placed in zinc formalin to prepare paraffin-embedded sections. One section from each sample was deparaffinized and stained with hematoxylin and eosin for histopathologic examination. A noninfected foreskin was also processed and sections were prepared.

Immunohistochemistry

Serial sections from each foreskin implant or the noninfected foreskin were deparaffinized and incubated overnight with a monoclonal antibody (1:50 dilution) to PCNA (Dako Corporation, Carpinteria, CA). An alkaline phosphatase-labelled, goat antimouse aniserum was then added, followed by developing. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used according to the manufacturer's instructions to detect antibody binding to PCNA in tissue sections. Sections were analyzed by light microscopy for evidence of staining indicating anti-PCNA binding.

DNA In Situ Hybridization

Sections of foreskin implants grown in athymic mice or noninfected foreskin were deparaffinized and processed with the Digene Tissue Hybridization Kit (Digene Diagnostics, Beltsville, MD) as recommended by the manufacturer. Sections from each tissue were deparaffinized with xylene and ethanol, heated for 5 minutes at 100°C, then hybridized for 18 hours at 37°C using a mixture of biotinylated probes for HPV types 6 and 11. Detection of hybridized probe was performed by incubation of slides with a streptavidin-alkaline phosphatase conjugate and reaction with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate. Cells positive for HPV DNA were identified by purple nuclear staining of epithelial cells in the foreskin implants.

RESULTS

Human foreskin implants were removed from pairs of mice at five time points, 1 week apart, beginning 4 weeks after HPV 11 infection and implantation. All implants were analyzed for HPV DNA and PCNA. HPV DNA was detected in all implants in the in situ hybridization assay. Initially, HPV DNA was seen in small foci of differentiated cells. The number of HPV DNA-positive nuclei increased at each time point until nearly all cells in the upper spinous and granular layers were HPV DNA-positive in the in situ hybridization assay.

PCNA was detected in the nucleus of cells in the HPV DNA-positive regions of the foreskin implants. In general, PCNA was detected in foci of cells. These foci were larger than, but included the foci of HPV DNA-positive cells. In the noninfected human foreskin tissue, PCNA was detected only in basal cells (not shown). PCNA staining in the noninfected foreskin tissue resembled that seen in HPV DNA-negative areas of the foreskin implants.

At 4 weeks, very small foci of HPV DNA-positive cells were present in differentiated cells (Fig. 1). In HPV DNA-negative regions of the implants, PCNA was detected only in basal cells. In contrast, PCNA was abundant in differentiated and undifferentiated cells in regions of HPV DNA-positive (Fig. 1). The epithelium was mildly thickened in the area of abundant PCNA-positivity. In addition, nuclei in these foci were slightly enlarged compared to nuclei of cells in HPV DNA-neg-

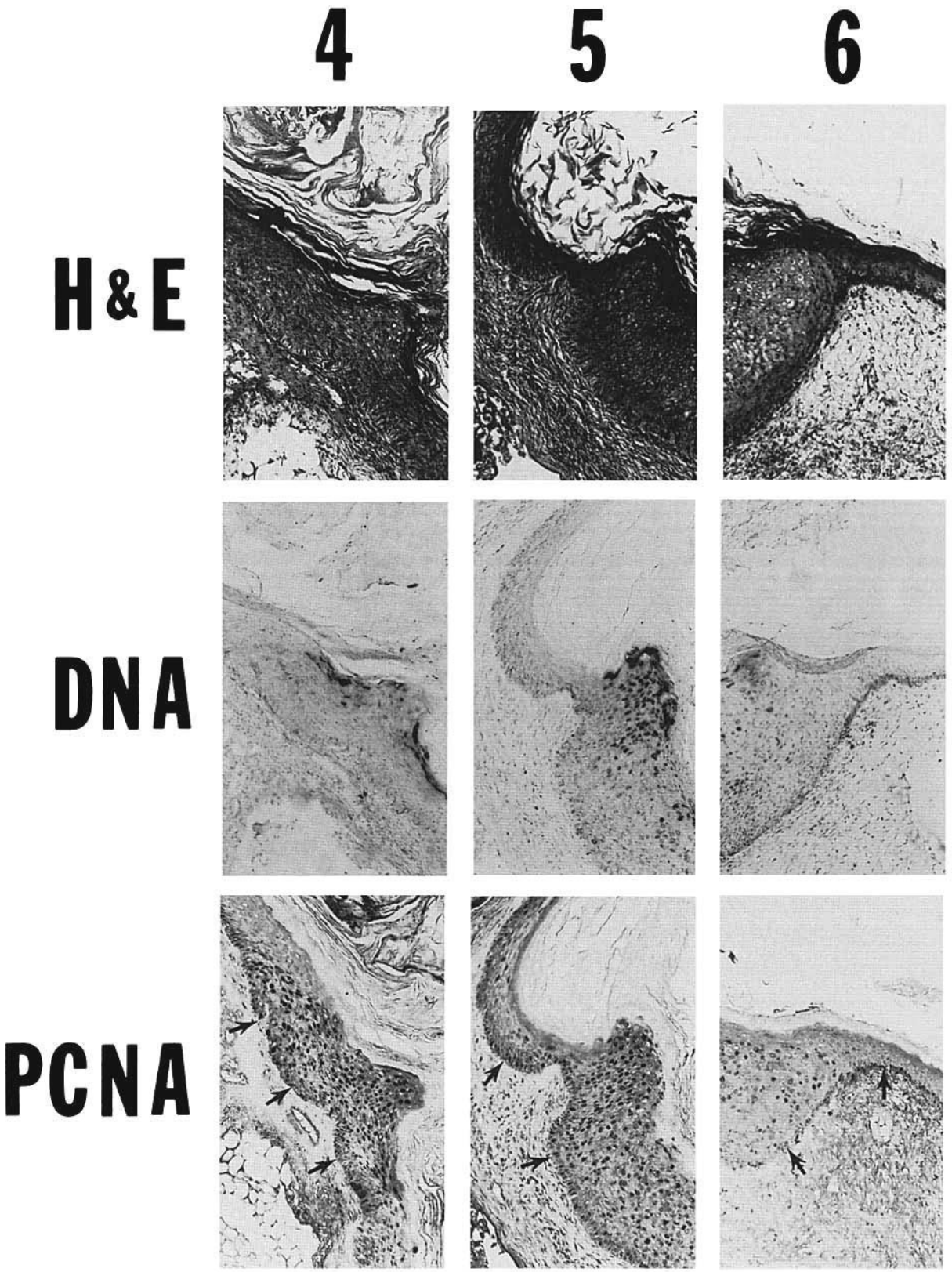

Fig. 1. Analysis of HPV 11-infected human foreskin implants grown in athymic mice and removed at 4, 5, or 6 weeks. Numbers at the top of the figure refer to the time in weeks that the implant shown was removed from the mouse. H & E: hematoxylin and eosin stain; DNA: in situ hybridization assay for HPV 11 DNA; PCNA: immunohistochemical analysis for PCNA. Arrows in the PCNA-stained sections point to the basal layer of the epithelium. Original magnification 100×.

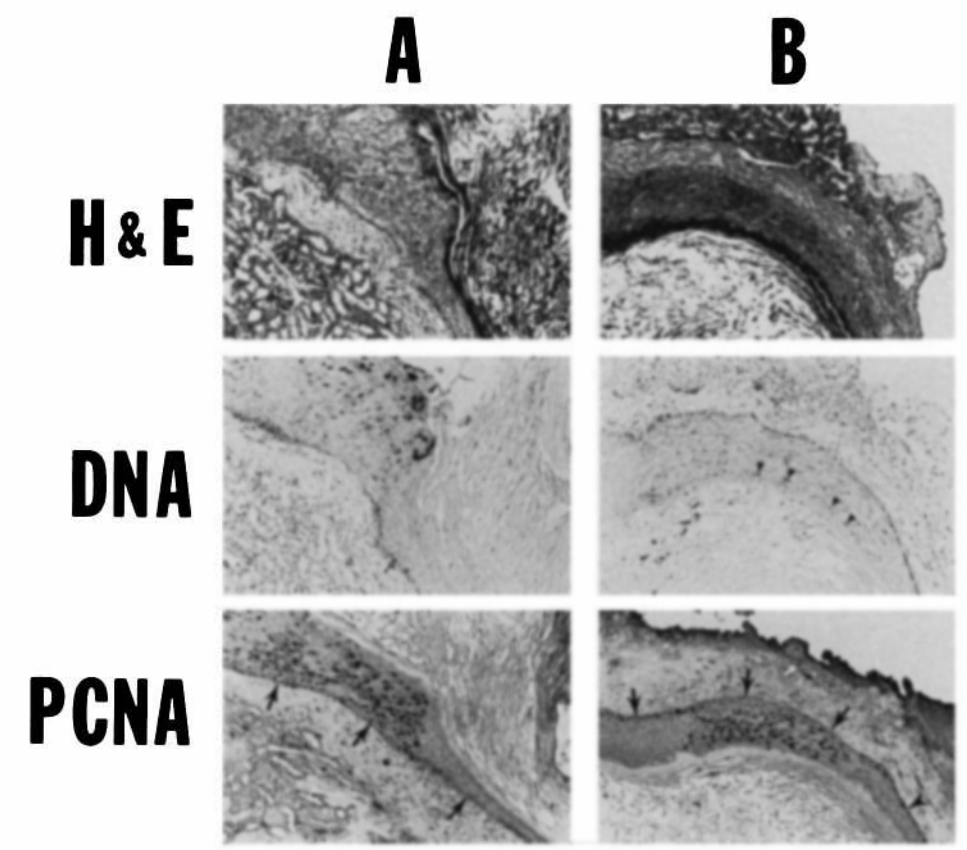

Fig. 2. Analysis of a single implant removed at 7 weeks. **A** and **B** indicate two separate areas of this implant, analyzed for histology, HPV DNA, and PCNA as in Figure 1. Arrows in the PCNA-stained sections point to the basal layer of epithelium. The arrowheads in B indicate several nuclei that were positive for HPV DNA in the in situ hybridization assay. Original magnification 100×.

ative areas. The implants removed at 5 weeks closely resembled the implants removed at 4 weeks, with small foci of HPV DNA-positive cells present in differentiated cells, and PCNA present throughout the epithelium in these regions (Fig. 1).

Implants removed at 6 weeks contained PCNA throughout the epithelium that also contained HPV DNA (Fig. 1). Histologic analysis of these areas revealed markedly thickened epithelium containing koilocytes (cells with abnormally shaped, enlarged nuclei and perinuclear halos).

Seven-week implants were similar to those removed at 6 weeks. In one implant, two separate foci of PCNA-positive cells was present (Fig. 2). The larger focus was in an area of strongly HPV DNA-positive granular layer cells and thickened epithelium. PCNA-positive cells were present in the upper spinous and granular layers of the infected epithelium in addition to undifferentiated cells. In the smaller focus of PCNA-positive cells, no histologic abnormalities were present, and only four HPV DNA-positive cells could be identified (Fig. 2).

At 8 weeks, virtually all of the epithelium was abnormally thickened, and extensive koilocytosis was present (Fig. 3). These implants histologically resembled mature condylomata acuminata. Cells in the upper spinous and granular layers were strongly positive for HPV DNA. In contrast, PCNA was most strongly detected in cells in the spinous layer and in basal cells. Koilocytes were noted to be somewhat less often PCNA-positive than cells in deeper epithelial layers.

Three implants (two removed at 5 weeks and one removed at 6 weeks) were completely negative for HPV DNA in the in situ hybridization assay (not shown). These implants were histologically identical to normal stratified epithelium. No cells in these implants were positive for HPV DNA. PCNA was present only in the basal cells, as was the case in HPV DNA-negative regions of implants shown in Figures 1 and 2 (illustrated by arrowheads).

DISCUSSION

In tissues known to be nonproliferative or to show low turnover, PCNA detection is minimal. The spatial distribution of PCNA immunoreactivity in normal proliferating tissues is precisely as expected for a proliferation marker. PCNA is generally detected in stratified squamous epithelia only in the basal layer of cells. Our time course studies demonstrate that PCNA can be detected in both differentiated and undifferentiated cells

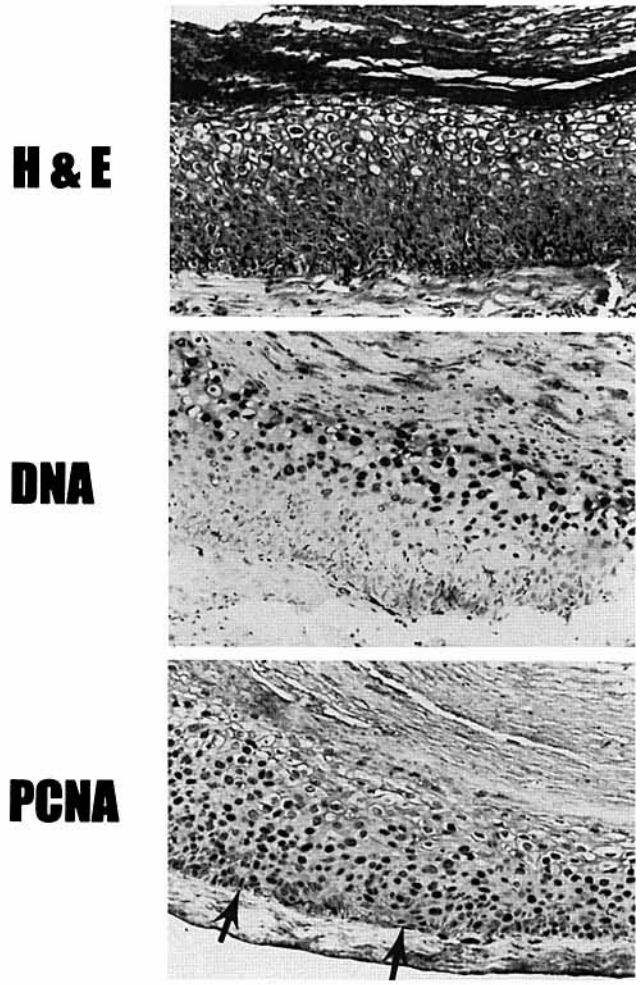

Fig. 3. Analysis of an implant removed at 8 weeks. The same studies were performed as indicated in Figure 1. Arrows in the PCNA-stained sections point to the basal layer of epithelium. Original magnification 100×.

of HPV-infected epithelium beginning 4 weeks after HPV 11 infection and implantation into athymic mice. The factors involved in PCNA induction by HPV have not been completely determined. Halbert et al. [1992] showed that the HPV 16 E7 protein, but not the HPV 6 E7 protein, can induce PCNA in raft cultures. When HPV 16 E6 was added to HPV 6 E7, PCNA was induced. Our data suggest that PCNA is being induced at a very early point in HPV infection, but we cannot prove that a direct effect of any HPV protein is causing this induction. However, obvious candidates for PCNA induction are the early gene products, such as E6 and E7, but other early genes may be important, such as E1 and E2. It has not been established that viral transcription is a necessary feature for PCNA induction. There is evidence that bovine papillomavirus (BPV) replication is influenced by PCNA, but a similar influence on HPV DNA replication remains unproven [Müller et al., 1994].

It is assumed that HPV initially infects basal cells and that infection spreads upward through the epithelium as cells differentiate. HPV then undergoes vegetative replication in these differentiated cells. If these assumptions are correct, it is puzzling that all layers of cells would appear to be producing PCNA in abundance early in the course of HPV infection. Our study suggests that PCNA is induced prior to significant replication of HPV DNA to a level detectable by in situ hybridization and that induction occurs in all layers of epithelium. Whereas the intensity of the PCNA signal remained relatively constant between early and late time points, the number of HPV DNA-positive cells gradually increased, suggesting that PCNA was induced to a level sufficient for detection in all epithelial layers early in HPV 11 infection. In implants removed at later time points, PCNA was most abundant in layers of epithelium just below those containing maximal HPV DNA.

Although PCNA and HPV DNA were detected in the same foci of cells in the foreskin implants, more cells were positive in these foci for PCNA than were HPV DNA-positive. PCNA was detected in sharply demarcated foci of cells, suggesting that an expanding clone of cells had been stimulated to produce PCNA and that all cells in this clone were producing this protein in abundance. We found PCNA induction prior to the appearance of abundant HPV DNA and in areas of normal or minimally abnormal histology. It is possible that some PCNA-positive but HPV DNA-negative cells contained a viral copy number below the threshold of sensitivity for in situ hybridization assay. Low levels of viral proteins may be responsible for PCNA induction in the absence of high levels of viral DNA.

The findings in the mouse xenograft model show that PCNA is induced in human foreskin infected with HPV 11, a "low-risk" HPV type. Demeter et al. [1994] found PCNA induction in condylomata acuminata infected with HPV types 6 or 11. These condylomata acuminata were likely to resemble our 8-week foreskin implants, because both contained epithelial thickening and abundant koilocytosis. Other studies have demonstrated PCNA induction by "high-risk" HPV types such as HPV 16. Using organotypic raft cultures derived from HPV types 16 and 18 immortalized keratinocyte cell lines, Merrick et al. [1992] demonstrated PCNA throughout the epithelium, compared to raft cultures free of HPV, which contained PCNA only in basal cells. These authors suggested that maintenance of a proliferative state, perhaps secondary to an effect of HPV 16 or 18 sequences, may contribute to development of malignancy. We would argue that a similar contribution is made by HPV 11 sequences, but the consequence of this contribution is benign epithelial proliferation.

In summary, PCNA can be detected early in the course of experimental HPV 11 infection. PCNA can be detected in foci of cells also containing HPV DNA. No foci of differentiated PCNA-positive cells were found in HPV DNA-negative regions of the implants. Likewise, HPV DNA was never found in regions of the foreskin

implants that did not contain suprabasal PCNA. Both differentiated and undifferentiated layers of HPV-infected epithelium contained detectable PCNA early in infection. In later stages of infection, when the implants histologically resemble mature condylomata acuminata lesions, PCNA is maximally detected in layers of epithelium below those containing the highest amounts of HPV DNA. The xenograft model provides an opportunity to observe PCNA induction in relation to specific events in the HPV replicative cycle.

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